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Evaluation of [^{67}Ga]-insulin for insulin receptor imaging

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Abstract

BACKGROUND: Radiolabelled human recombinant insulin can be used for the imaging of insulin receptors in some tumours where FDG has natural uptake and diminishes the value of its imaging.

MATERIAL AND METHODS: Insulin was successively labelled with [^{67}Ga]-gallium chloride after conjugation with freshly prepared cyclic DTPA-dianhydride (HPLC radiochemical purity assay > 96%) followed by biodistribution studies in normal rats, white blood cell labelling and preliminary SPECT studies.

RESULTS: *In vitro* studies demonstrated the retention of radiolabelled insulin receptor affinity using freshly prepared human white blood cells at different blood sugar conditions. Preliminary *in vivo* studies in a normal rat model was performed to determine the biodistribution of the radioimmunoconjugate at up to 44 h. SPECT images revealed high uptake of the liver.

CONCLUSION: Radiolabelled insulin is stable enough to be used in biological studies in order to image insulin receptors in diabetic conditions as well as possible tumour imaging applications. The data was consistent with other radiolabelled insulin studies.

Key words: insulin, gallium-67, radiolabelling, biodistribution, WBC binding, SPECT

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Introduction

The metabolism of glucose has been an interesting target for tumour imaging for three decades. The development of radiolabelled glucose by many research groups in the 1970s and 1980s presented the most common PET radiopharmaceutical, ^{18}F-FDG. Since then FDG has been used in the detection and staging of several malignancies. Few limitations have been proposed in the last few years in using FDG as a tracer, including natural accumulation of the tracer in the bladder, liver and brain, which can limit the detection of malignancies of these regions.

Insulin (Figure 1) has been radiolabelled with many SPECT and PET radionuclides such as ^{99m}Tc [1], ^{123}I [2, 3], ^{18}F [4, 5] for various purposes, such as the study of insulin biodistribution in diabetic subjects [6] and over-expression of insulin receptor on the surface of IM-9 lymphoblastoid tumour cells [7].

In order to obtain an insulin conjugate for use in diagnostic studies using metallic PET or SPECT radioisotopes, ^{67}Ga-labelled insulin was prepared for preliminary biodistribution studies, based on our recent experiences on the preparation of radiometal-labelled proteins [8]. Radiolabelled protein was injected intravenously into the tail vein of normal rats and the biodistribution of the tracer was checked among the tissues.

Material and methods

Production of ^{67}Ga was performed at the Nuclear Research Centre for Agriculture and Medicine (NRCAM) using a 30 MeV cyclotron (Cyclone-30, IBA). Enriched zinc-68 chloride with enrichment of > 95% was obtained from the Ion Beam Separation Department at NRCAM. Sephadex G-50, sodium acetate, phosphate buffer components, methanol and ammonium acetate were purchased from the Sigma-Aldrich Chemical Co. (U.K.). Cyclic DTPA dianhydride was freshly prepared and kept under a blanket of N_2. The insulin was a pharmaceutical sample purchased from Roche Co. and was used without further purification. Thin layer chromatography (TLC) of non-radioactive products was performed on polymer-backed silica gel (F 1500/LS 254, 20 × 20 cm, TLC Ready Foil, Schleicher & Schuell®). Mixtures of ammonium acetate/10%-methanol (50:50 or 90:10) were used as eluent. Radio-chromatography was performed by counting different 5 mm slices of polymer-backed silica gel paper using a high purity germanium (HPGe) detec-

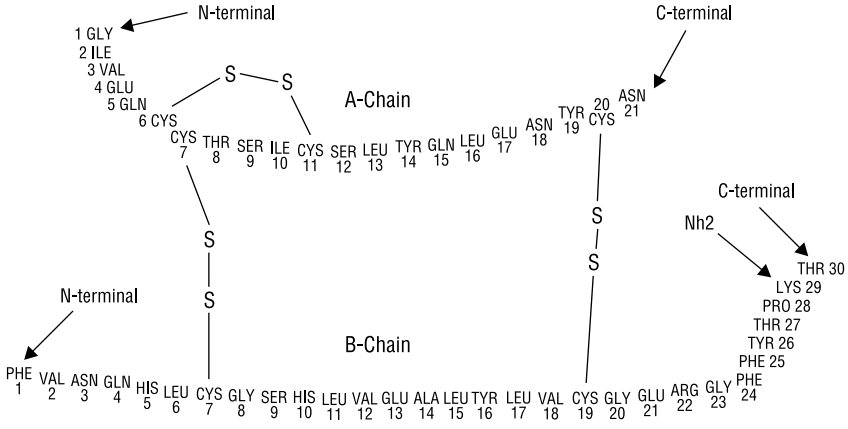

Figure 1. Structure of human insulin.

tor coupled with a Canberra™ (model GC1020–7500SL) multichannel analyzer. Calculations were based on the 184 keV peak for ^{67}Ga. All values were expressed as mean ± standard deviation (mean ± SD) and the data were compared using student T-test. Statistical significance was defined as $P < 0.05$. Animal studies were performed in accordance with the United Kingdom Biological Council's *Guidelines on the Use of Living Animals in Scientific Investigations,* 2nd edn.

Radiolabelling of the antibody conjugate with ^{67}Ga

The antibody DTPA-conjugate was labelled using an optimization protocol according to literature [9, 10]. Typically, 37–40 MBq of ^{67}Ga-chloride (in 0.2 M HCl) was added to a conical vial and dried under a flow of nitrogen. To the Ga-containing vial, conjugated fraction was added in 1 ml of phosphate buffer (0.1 M, pH = 8) and mixed gently for 30 seconds. The resulting solution was incubated at room temperature for 30 minutes. Following incubation, the radiolabelled antibody conjugate was checked using ITLC/HPLC methods for chemical and radiochemical purity. If the percentage of impurities was more than 10%, the sample was purified using gel filtration as follows. Briefly, the radiolabelled mixture was passed through a Sephadex G-50 column (2 × 15 cm, 2 g in 50 ml of Milli-Q® water) separately, and one-millilitre fractions were collected and checked for the presence of protein using UV absorbance at 280 nm or visible folin-phenol colorimetric assay. Control labelling experiments were also performed using $^{67}GaCl_3$, and DTPA with $^{67}GaCl_3$. High performance liquid chromatography (HPLC) was performed on the final preparation using acetate buffer solution (50 mM pH. 5.5) as eluent (flow rate: 1 ml/min pressure: 130 KgF/cm^2) for 20 min in order to elute low molecular weight components (Figure 2). Radiolabelled peptide was eluted using a gradient of the latter solution (100 to 0%) and citrate buffer solution (50 mM, pH.4, 0 to 100%) using reverse stationary phase.

Stability testing of the radiolabelled compound in presence of serum

Labelled compound stability in serum was assessed by gel filtration on a Sepharose column (1 × 30 cm). The column was equilibrated with PBS and eluted at a flow rate of 0.5 ml/min at room temperature; 0.5 ml fractions were collected.

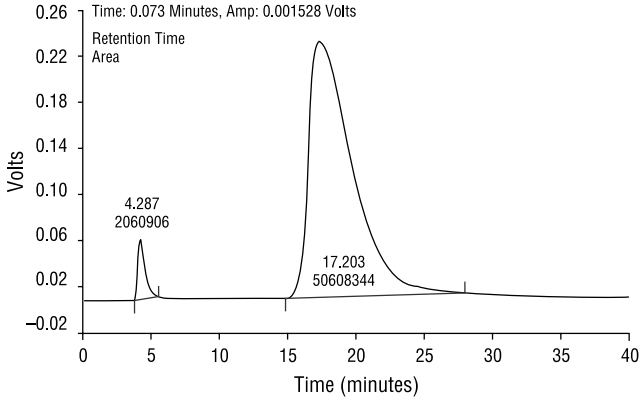

Figure 2. HPLC chromatogram of final radiolabelled solution on a reversed phase column using a gradient of acetate/citrate buffer.

White blood cell affinity experiment

Due to the adsorption of insulin in the surface of white blood cells for glucose consumption [11], these cells were used to determine the retention of insulin biological activity using the reported method for insulin cell affinity with slight modifications [2]. In a typical run, healthy male volunteer blood samples (3 ml) were collected in sterile polymer tubes with added anticoagulant. The samples were centrifuged at 3000 rpm for five minutes then the serum was discarded. The cell pellets were reconstituted in 1 ml of PBS followed by the addition of the ^{67}Ga-DTPA-insulin final solution (3 MBq). The samples were kept at 37°C for up to 2.5 hours. One-millilitre samples were taken at various time intervals (30, 60, 90, 150) and centrifuged at 3000 rpm for 5 min. The cell pellets were carefully washed with PBS and the washing solution was discarded. The area under the curve of the 184 keV peak was determined for samples (cell pellet and the supernatants) using an HPGe detector for 50 seconds and the ratio of cell/ /supernatants+cell was determined (n = 5).

Biodistribution of ^{67}Ga-DTPA-insulin in normal rats

To determine its biodistribution, ^{67}Ga-DTPA-insulin was administered to normal rats. A volume (50 μl) of final ^{67}Ga-DTPA--insulin solution containing 1.48 ± 0.07 MBq (40 ± 2 μCi) radioactivity was injected intravenously into rats through their tail vein.

The animals were sacrificed at exact time intervals (2, 3, 21.5 and 44 h), and the %ID/g of different organs was calculated as the percentage of injected dose (based on area under the curve of 184 keV peak) per gram using an HPGe detector.

Total blood cell labelling

The binding of the radiolabelled insulin to the superficial insulin receptors on the white blood cells was determined using a cell tagging protocol. Briefly, a blood sample (5 ml) was taken from a male volunteer followed by centrifugation at 3000 rpm for five minutes. The supernatant was decanted and the separated blood cell pellet was washed three times by PBS (3 × 5 ml, pH. 7.4). The pellet was then gently re-suspended in PBS at 37°C using mild shaking. Then, radiolabelled insulin (3.7 MBq, 50–100 μl) was added to the cell suspension and kept at body temperature in an incubator with mild shaking. At specific time intervals, one millilitre of the suspension was collected and centrifuged at 3000 rpm for five minutes and the radioactive counts for the supernatant, cell pellet and whole mixture were determined in a dose calibrator. The control tests were performed using free ^{67}Ga3+ and ^{67}Ga-DTPA to minimize the effect of any unspecific labelling in exactly the same manner. The uptake ratio was determined as the amount of centrifuged cell mixture radioactivity per total initial activity used.

SPECT imaging of ^{67}Ga-DTPA-insulin in normal rats

Images were taken at 24 and 48 hours after administration of the radiopharmaceutical by a dual-head SPECT system. The mouse-to-high energy septa distance was 12 cm. The useable field of view (UFOV) was 540 mm × 400 mm. The spatial resolution in the coincidence mode was 10 mm FWHM at the CFOV. Sixty-four projections were acquired for 30 seconds per view with a 64 × 64 matrix.

Results and discussion

Conjugation of insulin with DTPA cyclic di-anhydride and radiolabelling of insulin with ^{67}Ga

The labelling yield of ^{67}Ga-DTPA-insulin was studied in a wide range of insulin/DTPA ratios in order to optimize the process and to improve ^{67}Ga-DTPA-insulin performance *in vitro*.

The mixture was finally tested by HPLC in order to determine the radiochemical purity before administration to rodent models for biodistribution studies. Figure 3 shows the HPLC chromatogram of ^{67}GaDTPA and the final solution. The fast eluting component (4.28 min) was shown to be a mixture of free ^{67}Ga and ^{67}GaDTPA, which were washed out on reverse phase stationary phase. Both compounds are ionic, so they are eluted at the same retention time. The radiolabelled protein was finally washed out at 17.2 minutes.

Stability of radiolabelled protein in vitro

The stability of the radiolabelled protein in vitro was determined after a challenge with phosphate-buffered saline and serum. ITLC analysis showed that the proteins retained the radiolabel over a period of several hours, indicating that the Ga-protein chelate was of high affinity.

These results were confirmed by gel filtration chromatography. After incubation of [^{67}Ga]-DTPA-insulin with PBS for two hours, almost all of the radioactivity eluted in the same position as [^{67}Ga]-DTPA-insulin; there was no evidence for large-scale release of free Ga. Similarly, gel filtration chromatography of ^{67}Ga-DTPA-insulin after a two-hour incubation with human serum showed that the radioactivity still eluted in the same position. Thus, there was no evidence for either degradation or transchelation of ^{67}Ga to other serum proteins over a period consistent with the normal blood clearance time of insulin.

Biodistribution studies

The distribution of [^{67}Ga]-DTPA-insulin among tissues was determined for untreated rats. A volume (0.1 ml) of final [^{67}Ga]--DTPA-insulin solution containing 4.4–5.2 MBq radioactivity (6 μg protein in 100 μL) was injected into the dorsal tail vein. The total amount of radioactivity injected into each mouse was measured by counting the 1 ml syringe before and after injection in a dose calibrator with a fixed geometry. The animals were sacrificed by ether asphyxiation at selected times after injection (2, 3, 21.5 and 44 h), the tissues (blood, heart, spleen, kidneys, liver, intestine, stomach, lung, skin) and faeces were weighed and their accumulated activities were determined with a γ-ray

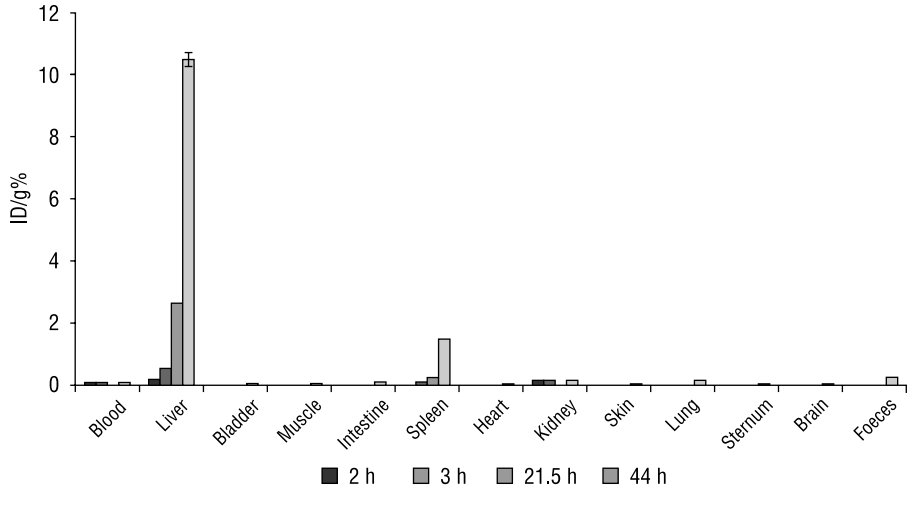

Figure 3. Percentage of injected dose per gram (%ID/g) of ^{67}Ga-DTPA-insulin in normal rat tissues 3–44 h post injection.

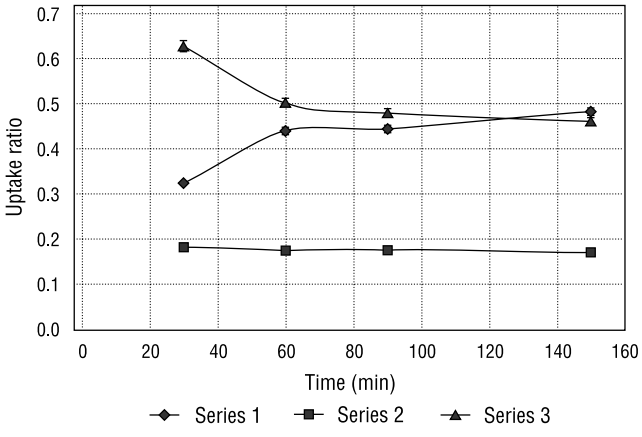

Figure 4. Radiolabelled insulin blood cell affinity determination as a function of time in normally fed (series 1), well fed (series 2) and fasting male volunteers (series 3).

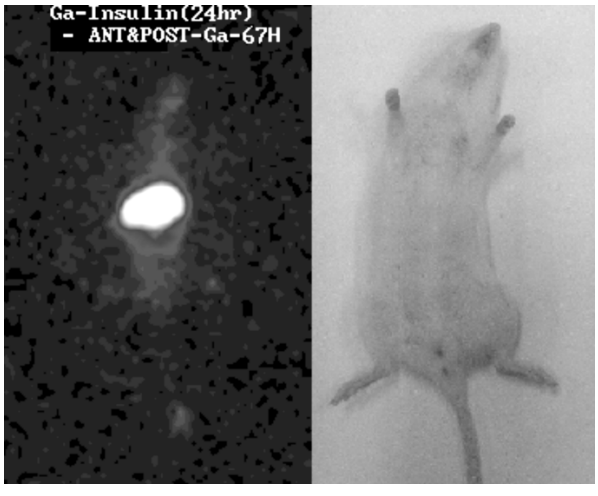

Figure 5. SPECT images of ^{67}Ga-DTPA-insulin in normal rat 24 hours post injection.

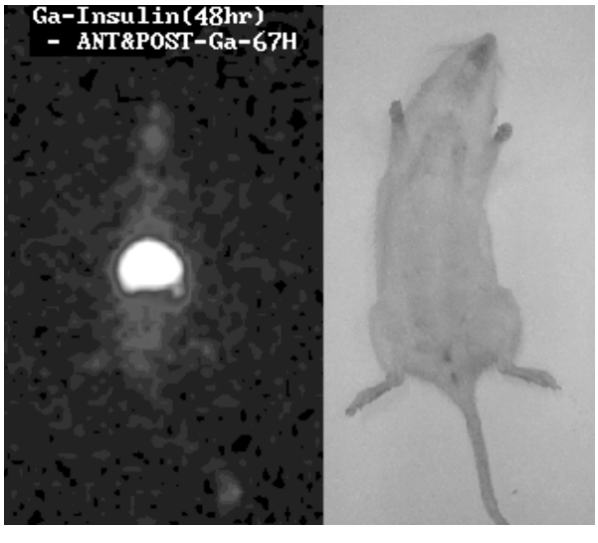

Figure 6. SPECT images of ^{67}Ga-DTPA-insulin in normal rat 48 hours post injection.

scintillation as a percent of the injected dose per gram of tissue (Figure 3).

In 2–3 hours, radiolabelled hormone is cleared from blood circle and most of the tracer accumulates in the liver. This is in agreement with reported ^{123}I-labelled insulin biodistribution results reported previously [12].

White blood cell labelling

The binding of the radiolabelled insulin to the superficial insulin receptors was determined using a cell tagging protocol. The cell binding of the tracer was significantly different in the three groups studied. In normally fed volunteers the cell uptake increased with time but reached a steady state after about 80 to 100 minutes. This can be explained by the fact that some of the unoccupied insulin receptors over the white blood cells were still ready for binding to the tracer. In well-fed volunteers (shown in square), the uptake does not change with the course of time, possibly due to a higher amount of native insulin secreted in the bloodstream of well-fed volunteers leading to the pre-occupation of all insulin receptors on the cell surface. In fasting volunteers, however, the results were unexpected: although the insulin receptors on the fasting cell surfaces seemed to be unoccupied, a drop in the cell uptake was observed in the course of time (Figure 4).

Imaging of [^{67}Ga]-insulin in normal rats

The best time period for scanning proved to be 24 to 48 hours post injection; up to two hours post injection a rather high accumulation was observed in the rat livers (Figure 5, 6). Interestingly, this data is comparable to other reported methods using various radionuclides to radiolabel insulin with the maximum uptake in the liver.

Conclusions

Total labelling and formulation of [^{67}Ga]-DTPA-insulin took about 60 minutes, with a yield of 99%. A suitable specific activity product was formed via insertion of ^{67}Ga cation. No unlabelled and/or labelled conjugates were observed upon RTLC analysis of the final preparations. The radio-labelled complex was stable in mice serum for at least 24 hours and no significant amount of free ^{67}Ga or ^{67}Ga-DTPA was observed. Trace amounts of ^{67}Ga-gallium chloride (≈ 1%) were detected by TLC. The final preparation was administered to normal rats, and the biodistribution of the radiopharmaceutical was checked one and six hours later. [^{67}Ga]-DTPA-insulin is a good probe for the imaging of insulin receptors.

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